

1 A multi-exon deletion within *WWOX* is associated with a 46,XY Disorder of Sex  
2 Development.

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28

29    **Abstract**

30    Disorders of sex development (DSD) are congenital conditions where chromosomal,  
31    gonad or genital development is atypical. In a significant proportion of 46,XY DSD  
32    cases it is not possible to identify a causative mutation, making genetic counseling  
33    difficult and potentially hindering optimal treatment. We present here the analysis of a  
34    46,XY DSD patient that presented at birth with ambiguous genitalia. Histological  
35    analysis of the surgically-removed gonads showed bilateral undifferentiated gonadal  
36    tissue and immature testis, both containing malignant germ cells. We screened genomic  
37    DNA from this patient for deletions and duplications using an Illumina whole genome  
38    SNP microarray. This analysis revealed a heterozygous deletion within the *WWOX* gene  
39    on chromosome 16, removing exons 6-8. Analysis of parental DNA showed that the  
40    deletion was inherited from the mother. cDNA analysis confirmed that the deletion  
41    maintained the reading frame, with exon 5 being spliced directly onto exon 9. This  
42    deletion is the first description of a germline rearrangement affecting the coding  
43    sequence of *WWOX* in humans. Previously described *Wwox* knockout mouse models  
44    showed gonadal abnormalities, supporting a role for *WWOX* in human gonad  
45    development.

46

47    Key words: Disorders of Sex Development, copy number, *WWOX*, gonad, microarrays.

48

49    **Introduction**

50    In the early stages of human embryogenesis the developing gonads are bipotent, being  
51    capable of forming either testes or ovaries. In males the expression of the Y  
52    chromosomal *SRY* gene initiates testis development, while ovarian development in

53 principle occurs only in the absence of *SRY* (reviewed in <sup>1</sup>). Following the establishment  
54 of sex-specific expression of key regulatory genes in the gonad, gonadal differentiation  
55 results in development of the external genitalia. As a result there are two main stages in  
56 gonad formation, and disruption of either can lead to disorders of sex development  
57 (DSD). DSD are surprisingly common, with ambiguous genitalia estimated to occur  
58 with an incidence of 1 in 4500 live births <sup>2</sup>.

59 A number of genes important in the regulation of sex determination have been  
60 identified, yet in as many as 70% of 46,XY DSD cases no genetic cause has been  
61 identified. We have previously demonstrated the power of whole genome copy number  
62 analysis with high density microarrays to identify causative mutations in DSD <sup>3,4</sup>. Here  
63 we describe the use of this approach to identify a multi-exon heterozygous deletion in  
64 the *WFOX* gene of a 46,XY DSD patient.

65

## 66 **Materials and Methods**

### 67 Array hybridization and analysis

68 Genomic DNA was hybridized onto an Illumina 610-Quad microarray at the Australian  
69 Genome Research Facility (Melbourne, Australia) following manufacturer's  
70 instructions. Data were analyzed using Genome Studio data analysis software  
71 (Illumina).

### 72 MLPA analysis

73 Deletion screening of the *WFOX* gene was performed with Multiplex Ligation-  
74 dependent Probe Amplification (MLPA). Probe design, the MLPA reaction and data  
75 analysis were performed as previously described <sup>5</sup>.

### 76 RNA extraction, cDNA generation and breakpoint PCR

77 RNA was extracted from lymphocytes obtained from the index case using standard  
78 procedures, with cDNA generated using random hexamers and the Transcriptor High  
79 Fidelity cDNA synthesis kit (Roche, Mannheim, Germany) according to the  
80 manufacturer's instructions. The PCR amplification across the deletion used the  
81 following primers; F – 5' CGAAACCGCCAAGTCTTTT 3', R – 5'  
82 CGTCTCTTCGCTCTGAGCTT 3', and was run under the following conditions:  
83 1 cycle: 60 seconds 95°C; 35 cycles: 30 seconds 95°C; 30 seconds 58°C; 60 seconds  
84 72°C; 1 cycle: 20 min 72°C.

#### 85 DNA Sequencing

86 Sanger sequencing was conducted at the Department of Pathology, University of  
87 Melbourne.

#### 88 Histological and Immunohistochemical Analysis

89 Research on human tissue samples was performed according to the Code for Proper  
90 Secondary Use of Human Tissue in The Netherlands, as developed by the Dutch  
91 Federation of Medical Scientific Societies (FMWV) version 2002, and approved by an  
92 institutional review board (MEC 02.981). Immunohistochemical detection of formalin-  
93 fixed paraffin-embedded tissue was performed for SOX9 and FOXL2 <sup>6</sup>, OCT3/4 <sup>7</sup>, and  
94 KITL <sup>8</sup>, as described previously.

95

## 96 **Results**

97 Physical examination of the index patient at the age of ten days revealed unfused  
98 labioscrotal folds, impalpable gonads, clitoral hypertrophy 20mm in size, and a perineal  
99 urogenital sinus. Genitography demonstrated the presence of a vagina and  
100 underdeveloped uterus. Chromosomal analysis showed a 46,XY karyotype with no

101 visible aberrations. Sequence analysis of the *SRY* and *NR5A1* genes did not reveal any  
102 variants, and MLPA analysis with a commercially available kit (MLPA P185-B1)  
103 containing probes targeted at the *WNT4*, *SRY*, *NR0B1*, *SOX9* and *NR5A1* genes did not  
104 show any deletions or duplications.

105 Small dysgenic gonads were present in the abdomen, and pathological analysis  
106 following complete removal at two years of age showed that they contained oedematous  
107 infantile testicular parenchyma (Figure 1). The epididymis was completely separated  
108 from the rete testis on both sides, and tubair epithelium was identified at the left side.  
109 The left gonad consisted of centrally located oedematous testicular tissue, positive for  
110 immunohistochemical detection of SOX9 (indicative for Sertoli cells) and negative for  
111 FOXL2 (a granulosa cell marker). A gradual transition towards undifferentiated gonadal  
112 tissue, containing both SOX9 and FOXL2 positive cells at the upper and lower poles  
113 were identified. Undifferentiated gonadal tissue is a gonadal pattern found specifically  
114 in patients with gonadal dysgenesis and is typically characterized by the combined  
115 expression of FOXL2 and SOX9 (usually with a preponderance of FOXL2), suggesting  
116 limited differentiation of the supportive cell lineage into pre-granulosa and pre-Sertoli  
117 cells<sup>9</sup>. Immature OCT3/4-positive germ cells were also found, either dispersed in  
118 ovarian-like stroma or organized together with Sertoli/granulosa cells in cord-like  
119 structures, reminiscent of sex cords. These structures have been recognized as the  
120 precursor lesion for gonadoblastoma<sup>10</sup>.

121 In spite of the presence of ovarian-like stroma, no ovarian follicles were present, and  
122 therefore the histology did not allow the diagnosis of ovarian differentiation. The right  
123 gonad displayed a similar morphology, with predominant, SOX9 positive testicular  
124 differentiation, except for the upper pole, where a large area of FOXL2 positive

undifferentiated gonadal tissue was seen, also containing scarce SOX9 positive cells. Based on morphological criteria and immunohistochemical analysis for OCT3/4 and TSPY (showing co-expression of both markers), presence of pre-malignant germ cells was diagnosed. The co-expression is assumed to identify the earliest pathogenetic changes in the genesis of malignant germ cell tumors. That the germ cells are indeed transformed was confirmed by staining for KITL. A limited hormonal work-up was carried out in the neonatal period, with normal values for ACTH, cortisol and 17 hydroxy progesterone. At 28 weeks, serum values were obtained for LH and FSH and were 1.0 and 5.2 U/l respectively. Although FSH levels were not measured prior to this, the relatively high value at this age suggests a higher value (in the range of gonadal dysgenesis) during full mini-puberty. A HCG test performed at 18 months shows a moderate rise of testosterone (testosterone 231 ng/dl after HCG, 500U, 2x/week for 3 weeks).

Microarray analysis did not reveal deletions or duplications covering any genes already known to be involved in DSD. The most promising candidate rearrangement was a 767 kb deletion on chr 16 (chr16:76956767-77723905; hg18) (Figure 2a). This deletion was located within the *WWOX* gene, and removed exons 6-8. MLPA probes for each of the nine *WWOX* exons were designed, and MLPA analysis confirmed that the deletion was restricted to these three exons (data not shown). The deletion is predicted to generate an alternative, in-frame mRNA product, with exon 5 being spliced directly onto exon 9. This alternative transcript was verified by sequencing cDNA derived from matched lymphocytes (Figure 2b).

148 MLPA analysis of parental DNA samples revealed that the deletion was maternally  
149 inherited, with no evidence of mosaicism<sup>11</sup>. The clinical history of the mother included  
150 a relatively late menarche (16 yrs) with irregular menstruation up to the first pregnancy.  
151 Prior to this pregnancy she had received hormonal stimulation, but this was stopped  
152 prior to conception. Four subsequent pregnancies occurred naturally. DNA analysis of  
153 the children (two brothers and two sisters, all unaffected) showed that none had  
154 inherited the deletion. Analysis of the *WWOX* gene in the index case did not show any  
155 sequence variants.

156

## 157 **Discussion**

158 We report here the first germline rearrangement affecting the coding sequence of the  
159 *WWOX* gene. *WWOX* consists of 9 exons, and is >1 Mb in size. It is located on the long  
160 arm of chromosome 16 in a known fragile site frequently rearranged in a wide range of  
161 cancers<sup>12</sup>, and is a suspected tumour suppressor gene<sup>13</sup>.

162 *WWOX* encodes a 414 aa, 46 kDa protein<sup>14</sup>. At the amino terminus there are two WW  
163 domains, with a short chain oxidoreductase (SDR) domain within the central portion of  
164 the protein<sup>15</sup>. The WW domains are believed to be involved in protein-protein  
165 interactions, including a number of transcription factors<sup>16, 17</sup>. The SDR region is  
166 thought to play a role in steroid metabolism<sup>18</sup>, with the exon 6-8 deletion identified in  
167 this study maintaining the reading frame but effectively ablating the SDR domain from  
168 the predicted protein (Figure 2c).

169 Two different *Wwox* knock-out mouse models have shown gonadal abnormalities,  
170 including defects of Leydig cell function in the testis<sup>18, 19</sup>. There are various possible  
171 mechanisms for *WWOX*-mediated testicular dysfunction. *WWOX* expression inhibits

172 the Wnt/ $\beta$ -catenin pathway in a dose dependent manner, although this inhibition is  
173 reduced if the SDR domain is removed <sup>20</sup>. The Wnt/ $\beta$ -catenin pathway is involved in  
174 normal ovarian development, and increased Wnt/ $\beta$ -catenin activity in the developing  
175 gonad through compromised WWOX activity may interfere with normal testis  
176 development. Indeed, expression of a stabilized form of  $\beta$ -catenin in the somatic cells of  
177 XY mice leads to male to female sex reversal <sup>21</sup>.

178 WWOX expression is high in endocrine tissues such as pituitary, testis and ovary <sup>22</sup>, and  
179 it may have a role in gonadotrophin or sex-steroid biosynthesis. It has been suggested  
180 that a truncated WWOX protein consisting of both WW domains but without a  
181 functional SDR domain may act as a competitor of the full-length protein. The truncated  
182 WWOX could bind to WWOX-interacting proteins via the WW domains, but would not  
183 have any SDR activity <sup>20</sup>.

184 Although the mother of the index case carried the same deletion there was a milder  
185 gonadal phenotype, with late menarche. An increase in activity of the Wnt/ $\beta$ -catenin  
186 pathway due to reduced WWOX activity would not necessarily be expected to have an  
187 effect on ovarian development, as this pathway is already active during this process.  
188 There may however be a disturbed ovarian function due to impaired steroidogenesis, as  
189 was observed in one of the mouse models <sup>18</sup>.

190 In conclusion, we have identified a multi-exon deletion in *WWOX* in a 46,XY DSD  
191 patient with ambiguous genitalia. This finding is the first germline rearrangement  
192 affecting *WWOX* coding sequence described in humans, and implicates *WWOX* in  
193 normal gonadal development.

194

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201 Conflict of Interest Statement

202 The authors declare no conflict of interest.

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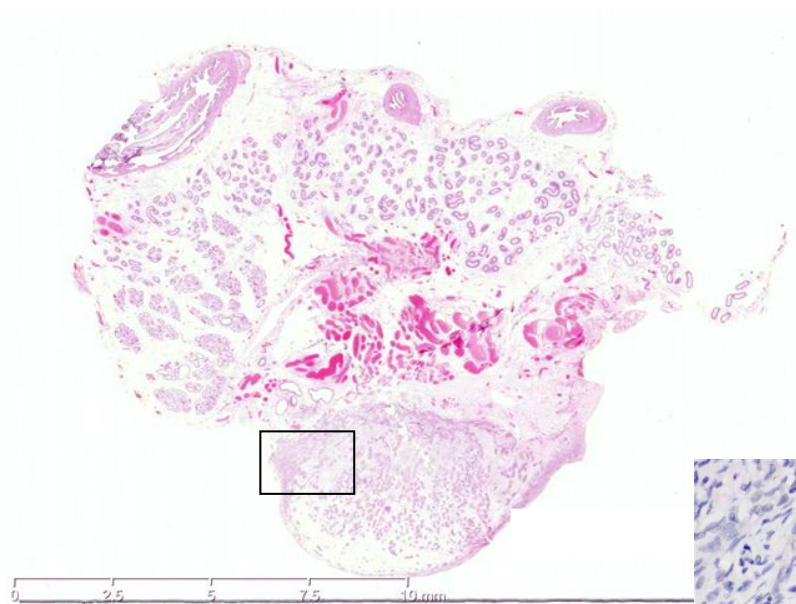
204 **Figure 1.** Representative histological and immunohistochemical findings for the left  
205 gonad: A) total overview of the gonad histology (H & E); B) higher magnification (2.5x  
206 magnification, indicated by square in A); immunohistochemical detection of C) SOX9,  
207 positive in the Sertoli cells; D) FOXL2, positive in the granulosa cells; E) OCT3/4; F)  
208 TSPY; G) KITL, all positive in the transformed germ cells. All immunohistochemical  
209 images are at the magnification of 100x, except G, being 50x.

210  
211 **Figure 2.** Molecular analysis of DNA from the 46,XY DSD patient. (A). The deletion  
212 identified by microarray analysis, removing 767 kb of genomic DNA on chromosome  
213 16. Data are plotted along the chromosome, with each point representing the copy  
214 number estimate of an individual probe. The breakpoints of the deletion are shown by  
215 the broken vertical lines. (B) PCR analysis of the deletion. cDNA was derived from  
216 lymphocytes of the index patient, and primers were designed to amplify a PCR product  
217 across the predicted breakpoint. Sequence analysis shows that exon 5 is spliced directly  
218 onto exon 9. (C) Effect of the deletion on the WWOX protein. The full length WWOX  
219 protein has two WW domains at the N-terminal, and a short chain oxidoreductase  
220 (SDR) domain at the C-terminal. A deletion of exons 6-8 is predicted to result in an in-  
221 frame but shortened product, with the SDR domain largely missing.

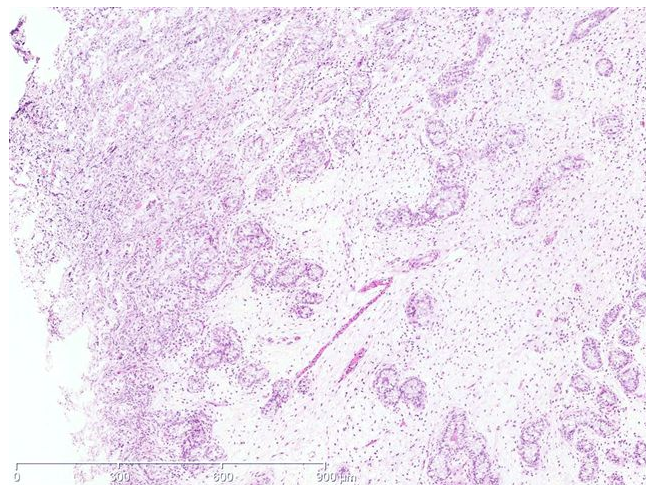
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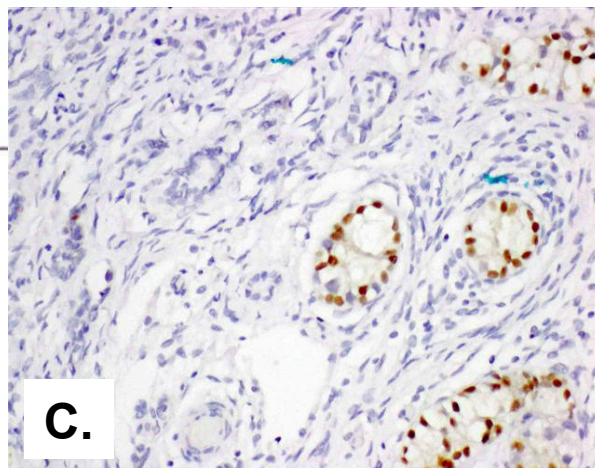




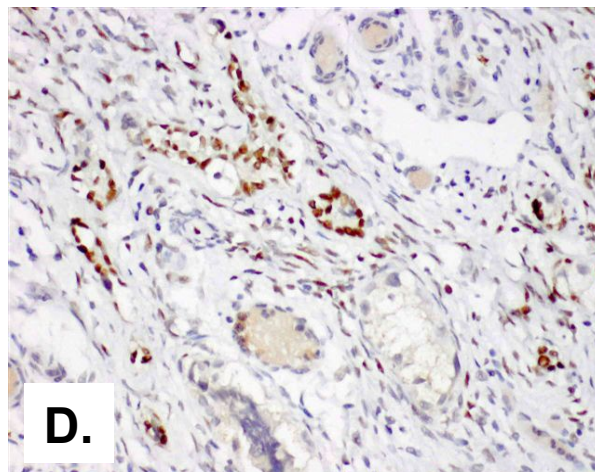
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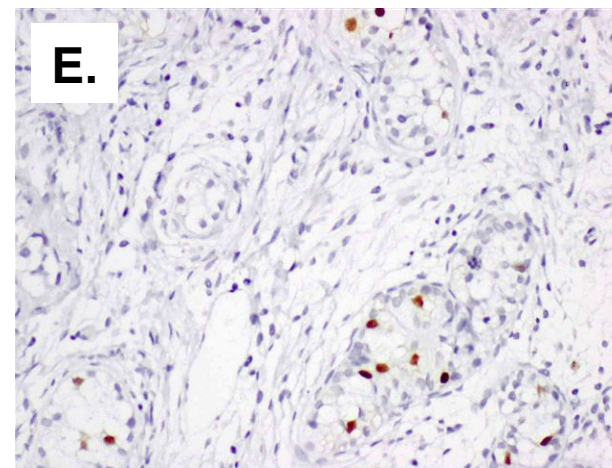
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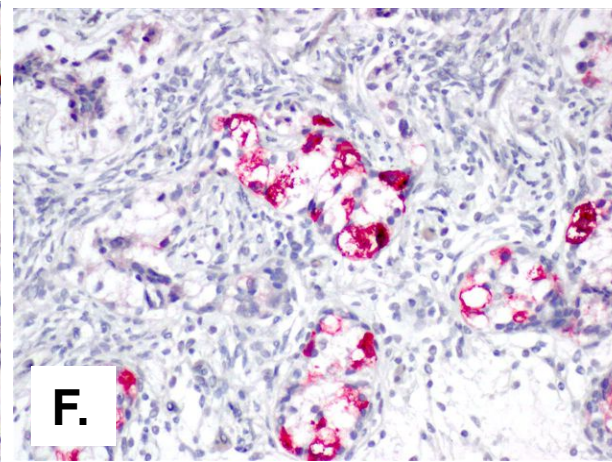
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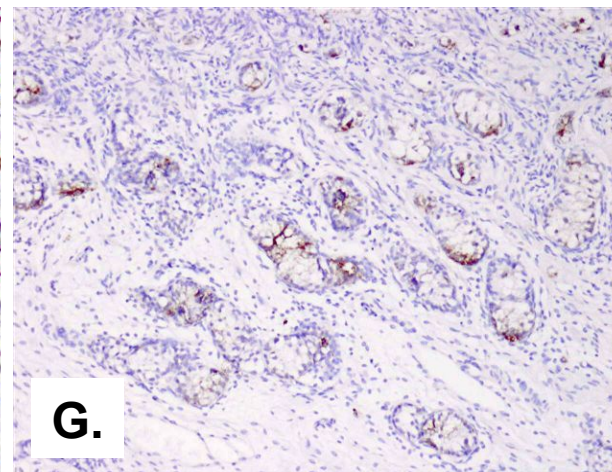
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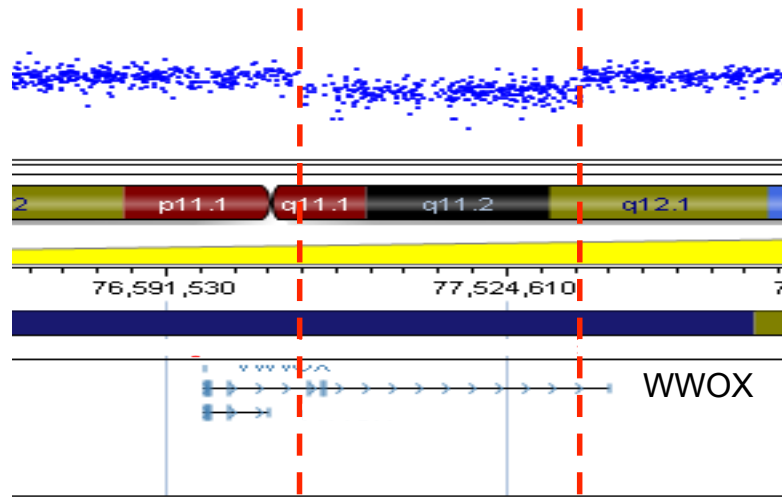


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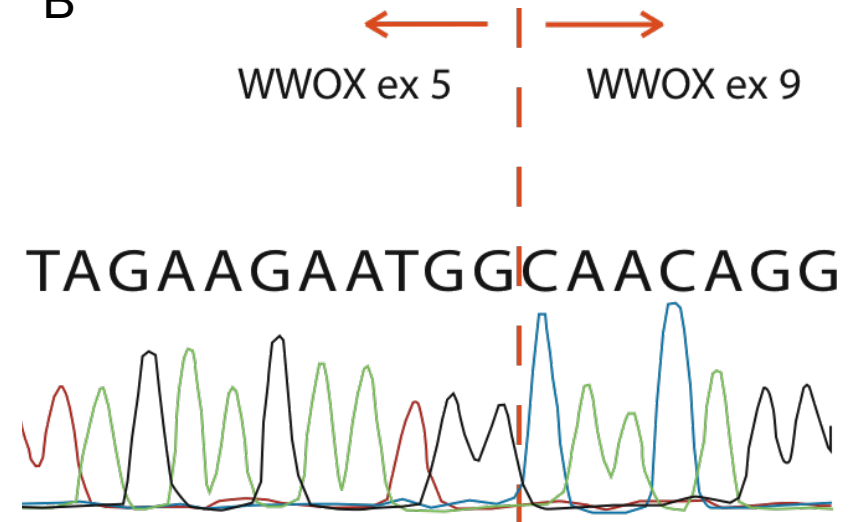


**G.**

A



B



C

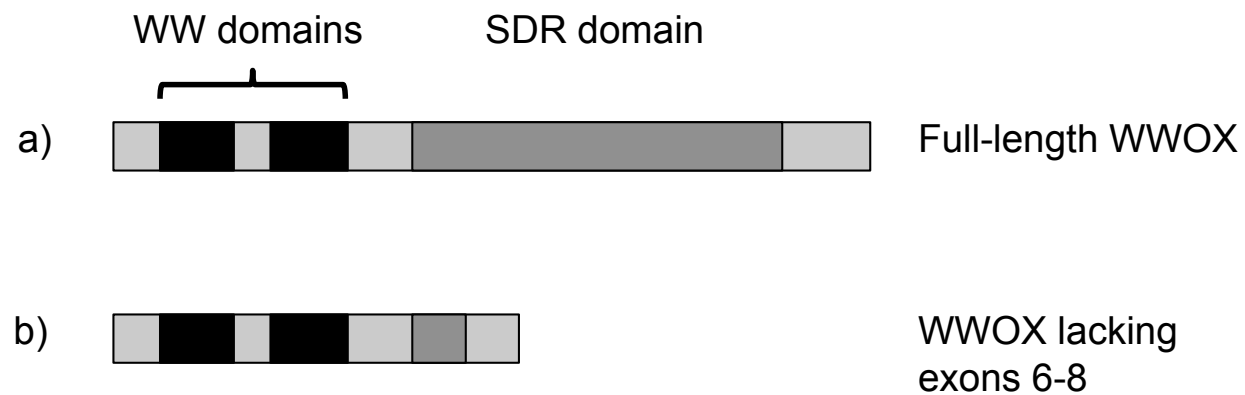


Figure 2